

Cytotoxic T Cell Responses to Human Telomerase Reverse Transcriptase in Patients With Hepatocellular Carcinoma

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Human telomerase reverse transcriptase, hTERT, has been identified as the catalytic enzyme required for telomere elongation. hTERT is expressed in most tumor cells but seldom expressed in most human adult cells. It has been reported that 80% to 90% of hepatocellular carcinomas (HCCs) express hTERT, making the enzyme a potential target in immunotherapy for HCC. In the current study, we identified hTERT-derived, HLA-A*2402–restricted cytotoxic T cell (CTL) epitopes and analyzed hTERT-specific CTL responses in patients with HCC. Peptides containing the epitopes showed high affinity to bind HLA-A*2402 in a major histocompatibility complex binding assay and were able to induce hTERT-specific CTLs in both hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice and patients with HCC. The CTLs were able to kill hepatoma cell lines depending on hTERT expression levels in an HLA-A*2402–restricted manner and induced irrespective of hepatitis viral infection. The number of single hTERT epitope-specific T cells detected by ELISPOT assay was 10 to 100 specific cells per 3×10^5 PBMCs, and positive T cell responses were observed in 6.9% to 12.5% of HCC patients. hTERT-specific T cell responses were observed even in the patients with early stages of HCC. The frequency of hTERT/tetramer⁺CD8⁺ T cells in the tumor tissue of patients with HCC was quite high, and they were functional. **In conclusion**, these results suggest that hTERT is an attractive target for T-cell–based immunotherapy for HCC, and the identified hTERT epitopes may be valuable both for immunotherapy and for analyzing host immune responses to HCC. (HEPATOLOGY 2006;43:1284–1294.)

Hepatocellular carcinoma (HCC) is the most frequent primary malignancy of the liver and has gained much clinical interest because of its increasing incidence.^{1–3} Although current advances in therapeutic modalities have improved the prognosis

of HCC patients,^{4–6} the survival rate is still not satisfactory. One of the reasons for the poor prognosis is the high rate of recurrence after treatment. To protect against recurrence, tumor antigen-specific immunotherapy is an attractive strategy. Although many tumor-specific antigens have been identified in various cancers, the number of HCC-specific antigens known is still limited.

Human telomerase reverse transcriptase, hTERT, has been identified as the catalytic enzyme required for telomere elongation.^{7–10} Recently, several results regarding hTERT-specific cytotoxic T cell (CTL) responses were reported for humans and mice.^{11–20} These reports revealed that hTERT-specific CTLs induced by stimulation with peptides or DNA-based immunization kill cancer cell lines that have high levels of hTERT, suggesting that hTERT-reactive T cell clones are not deleted from the human T cell repertoire and that hTERT may be a useful tumor-specific antigen as a target for T-cell–based immunotherapy for cancers. However, the existence of hTERT-specific CTLs and the relationship between immunological

Abbreviations: HCC, hepatocellular carcinoma; hTERT, human telomerase reverse transcriptase; CTL, cytotoxic T cell; HIV, human immunodeficiency virus; PBMC, peripheral blood mononuclear cell; AFP, alpha-fetoprotein; CMV, cytomegalovirus; FCS, fetal calf serum; TIL, tumor infiltrating lymphocyte; PCR, polymerase chain reaction; TRAP, telomerase repeat amplification protocol; IFN- γ , interferon gamma.

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responses and clinical factors have not been well characterized in patients with HCC.

In the current study, we first attempted to identify HLA-A*2402–restricted T cell epitopes derived from hTERT and then analyzed hTERT-specific immunological responses in HCC patients.

Patients and Methods

Patient Population. The study examined 72 HLA-A24–positive patients with HCC who were admitted to Kanazawa University Hospital between January 2002 and December 2004, consisting of 48 men and 24 women ranging from 46 to 81 years of age with a mean age of 67 ± 9 years. HCCs were detected by imaging modalities such as dynamic computed tomography (CT) scan, magnetic resonance imaging, and abdominal arteriography. The diagnosis of HCC was histologically confirmed by taking ultrasound-guided needle biopsy specimens in 29 cases, surgical resection in four cases, and autopsy in four cases. For the remaining 35 patients, the diagnosis was based on typical hypervascular tumor staining on angiography in addition to typical findings, which showed hyperattenuated areas in the early phase and hypoattenuation in the late phase on dynamic CT.²¹ All subjects were negative for antibodies to human immunodeficiency virus (HIV) and gave written informed consent to participate in this study in accordance with the Helsinki declaration. Eleven healthy blood donors with HLA-A24, who did not have a history of cancer and were negative for hepatitis B surface antigen and anti-HCV antibody, served as controls.

Laboratory and Virologic Testing. Blood samples were tested for hepatitis B surface antigen and HCV antibody by commercial immunoassays (Fuji Rebio, Tokyo, Japan). HLA-based typing of peripheral blood mononuclear cells (PBMCs) from patients and normal donors was performed using complement-dependent microcytotoxicity with HLA typing trays purchased from One Lambda.

The serum alpha-fetoprotein (AFP) level was measured by enzyme immunoassay (AxSYM AFP, Abbott Japan, Tokyo, Japan), and the pathological grading of tumor cell differentiation was assessed according to the general rules for the clinical and pathological study of primary liver cancer.²² The severity of liver disease (stage of fibrosis) was evaluated according to the criteria of Desmet et al.,²³ using biopsy specimens of liver tissue, where F4 was defined as cirrhosis.

Synthetic Peptides. To identify potential HLA-A24–binding peptides within hTERT, the sequence was reviewed using a computer-based program, which was

employed by accessing the World Wide Web site Bioinformatics and Molecular Analysis Section for HLA peptide binding predictions (available from <http://bimas.cit.nih.gov>). The HLA-A24–restricted epitopes derived from HIV envelope protein,²⁴ cytomegalovirus (CMV) pp65,²⁵ and HCV NS3 were used as control peptides to test for T cell responses, and the HLA-A2–restricted epitope derived from AFP²⁶ was used as a control peptide for HLA-A24 stabilization assay as previously described. Peptides were synthesized at Mimotope (Melbourne, Australia) and Sumitomo Pharmaceuticals (Osaka, Japan). They were identified using mass spectrometry, and their purities were determined to be greater than 80% by analytical high-pressure liquid chromatography.

Cell Lines. Three human hepatoma cell lines, HepG2, HuH6, and HuH7, were cultured in Dulbecco's minimum essential medium (Gibco, Grand Island, NY) with 10% fetal calf serum (FCS) (Gibco).

T2-A24 cells, which were T2 cells transfected with HLA-A*2402,²⁵ were cultured in RPMI 1640 medium containing 10% FCS and 800 $\mu\text{g}/\text{mL}$ G418 (GibcoBRL, Grand Island, NY). The HLA-A*2402 gene-transfected C1R cell line (C1R-A24)²⁷ was cultured in RPMI 1640 medium containing 10% FCS and 500 $\mu\text{g}/\text{mL}$ of hygromycin B (Sigma, St Louis, MO), and K562 was cultured in RPMI 1640 medium containing 10% FCS. All media contained 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin (GibcoBRL, Grand Island, NY).

Plasmid Construction. The plasmid which contains hTERT cDNA was subcloned as previously described.²⁸ In brief, the EcoRI-SalI fragment containing the hTERT cDNA was subcloned from pCI-Neo-hTERT, which was provided by Dr. Seishi Murakami (Cancer Research Institute, Kanazawa University). The fragment was subcloned into the EcoRI-SalI sites of the plasmid pNKZ-FLAG (pNKZ-FLAG-hTERT).

Injection of hTERT cDNA Into HLA-A*2402/K^b Transgenic Mice. Transgenic mice expressing the $\alpha 1$ and $\alpha 2$ domains from the HLA-A*2402 molecule and the $\alpha 3$ domain from the murine H-2K^b molecule,²⁹ kindly provided by Sumitomo Pharmaceuticals (Osaka, Japan), were bred in a specific-pathogen-free environment at the animal facility in Kanazawa University. For immunization with the hTERT cDNA, mice were injected with 50 μL cardiotoxin (Latoxan, Rosans, France) (10 $\mu\text{mol}/\text{L}$) per leg into the tibialis anterior muscles on both sides. Five days after injection of the cardiotoxin, the vector pNKZ-FLAG-hTERT containing the hTERT cDNA was injected into the same part of the muscle. Mice immunized with the plasmid pNKZ-FLAG were also used as negative controls. Splenocytes harvested on day 7 after the

Table 1. Characteristics of the Patients Studied

Clinical Diagnosis	No. of Patients	Sex M/F	Age (yr) Mean \pm SD	ALT (IU/L) Mean \pm SD	AFP (ng/mL) Mean \pm SD	Etiology (B/C/Others)	Child-Pugh (A/B/C)	Diff. degree* (Wel/Mod/Por/ND)	Tumor size** (Large/Small)	Tumor multiplicity (Multiple/Solitary)	Vascular Invasion (+/-)	TNM Stage (I/II/III/IIIB/IIIC/IV)
HCC patients	72	48/24	67 \pm 9	66 \pm 36	1722 \pm 7029	9/59/4	43/25/4	15/21/1/35	44/28	39/33	15/57	30/26/9/1/2/4
Normal donors	11	8/3	35 \pm 2	ND	ND	ND	ND	ND	ND	ND	ND	ND

*Histological degree of HCC; wel: well differentiated, mod: moderately differentiated, por: poorly differentiated, ND: not determined.

**Tumor size was divided into either "small" (\leq 2 cm) or "large" ($>$ 2 cm).

injection of cDNA were tested directly *ex vivo* for IFN- γ production using an ELISPOT assay.

Preparation of PBMCs and Tumor-Infiltrating Lymphocytes. PBMCs were isolated as previously described.^{30,31} Fresh PBMCs were used for the CTL assay, and the remaining PBMCs were resuspended in RPMI 1640 medium containing 80% FCS and 10% dimethyl sulfoxide (Sigma, St. Louis, MO) and cryopreserved until used. Tumor-infiltrating lymphocytes (TILs) were isolated by mechanical homogenization of tumors, which were resected by surgical treatment and cryopreserved as described until used.

Major Histocompatibility Complex Binding Assay. Peptide binding assays were performed as previously described.^{31,32} The data were expressed as % mean fluorescence intensity (MFI) increase, which was calculated as follows: Percent MFI increase = (MFI with the given peptide - MFI without peptide)/(MFI without peptide) \times 100.

ELISPOT Assay. ELISPOT assays were performed as previously described³¹ with the following modifications. Three hundred thousand unfractionated PBMCs or 100,000 TILs with 10,000 T2-A24 cells were added in duplicate cultures of RPMI 1640 medium containing 5% FCS together with the peptides at 10 μ g/mL. For the mouse assay, 2 \times 10⁵ spleen cells were used for each well. The number of specific spots was determined by subtracting the number of spots in the absence of antigen from that in the presence of antigen. Responses were considered positive for the human ELISPOT assay if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than that in the absence of antigen.

Cytotoxicity Assay. hTERT-derived peptide-specific T cells were expanded from PBMCs in 96-well round-bottomed plates (NUNC, Naperville, IL) as previously described.³⁰ Briefly, 400,000 cells per well were stimulated with 10 μ g/mL synthetic peptide, 10 ng/mL rIL-7, and 100 pg/mL rIL-12 (Sigma) in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum, 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cultures were re-stimulated with 10 μ g/mL peptide, 20 U/mL of rIL-2 (Sigma) and 1 \times 10⁵ mytomicin C-treated autologous PBMCs on days 7 and 14. On

days 3, 10, and 17, 100 μ L RPMI with 10% human AB serum and 10 U/mL rIL-2 (final concentration) were added to each well. Cytotoxicity assays were performed as previously described.³¹

Tetramer Staining and Flow Cytometry. Peptide hTERT₄₆₁-specific tetramer was purchased from Medical Biological Laboratories Co., Ltd (Nagoya, Japan). Tetramer staining was performed as previously described.³³ In brief, PBMCs and TILs were stained with CD4-FITC, CD14-FITC, CD19-FITC, CD8-PerCP (BD PharMingen, San Diego, CA), and tetramer-PE (10 μ L) for 30 minutes at room temperature. Cells were washed, fixed with 0.5% paraformaldehyde/phosphate-buffered saline, and analyzed on a FACSCalibur flow cytometer. Data analysis was undertaken with CELLQuest software (Becton Dickinson, San Jose, CA).

Telomerase Assay. Telomerase activity was measured by two methods according to the manufacturer's directions. First, a polymerase chain reaction (PCR)-based telomerase repeat amplification protocol (TRAP) assay was carried out with a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd., Auckland, New Zealand). The products of the PCR were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR-Green I (Molecular Probes, Eugene OR). Second, a TRAP enzyme-linked immunosorbent assay (ELISA) was used to quantitatively measure telomerase activity with a TRAPEZE ELISA telomerase detection kit (Intergen Co. Ltd.). Cell extracts were prepared from HepG2, HuH6, and HuH7 cells and used at 0.01 μ g per assay. Telomerase activity was also measured in the tumor of 10 patients with HCC who received surgical treatment. Cell extracts were prepared from resected tumors and used at 0.1 μ g per assay.

Statistical Analysis. Fisher's exact test (2-sided *P*-value) and the unpaired Student's *t* test were used to analyze the effect of variables on immune responses in HCC patients.

Results

Patient Profiles. The clinical profiles of the patients are shown in Table 1. The tumors of 37 patients were

Table 2. Peptides

Peptide	Source	Start Position	Amino Acid Sequence	HLA Restriction	Score*
hTERT ₁₀₈₈	hTERT	1088	TYVPLLGSL	HLA-A24	432
hTERT ₈₄₅	hTERT	845	CYGD MENKL	HLA-A24	317
hTERT ₁₆₇	hTERT	167	AYQVCGPPL	HLA-A24	300
hTERT ₄₆₁	hTERT	461	VYGFVRACL	HLA-A24	280
hTERT ₃₂₄	hTERT	324	VYAETKHFL	HLA-A24	240
hTERT ₁₀₀₉	hTERT	1009	AYRFHACVL	HLA-A24	200
hTERT ₃₈₅	hTERT	385	RYWQMRPLF	HLA-A24	200
hTERT ₆₃₇	hTERT	637	DYVVGARTF	HLA-A24	150
hTERT ₆₂₂	hTERT	622	RFIPKPDGL	HLA-A24	72
hTERT ₈₆₉	hTERT	869	DFLLVTPHL	HLA-A24	42
HIV enV ₅₈₄	HIV envelope	584	RYLRDQQLL	HLA-A24	720
CMV pp65 ₃₂₈	CMV pp65	328	QYDPVAALF	HLA-A24	168
HCV NS3 ₁₀₃₁	HCV NS3	1031	AYSQQTRGL	HLA-A24	200
AFP ₁₃₇	AFP	137	PLFQVPEPV	HLA-A2	3

*Estimated half-time of dissociation from the HLA-A24 or -A2 allele (min).

histologically classified as 15 well, 21 moderately, and 1 poorly differentiated HCC. Other patients were diagnosed with HCC based on typical CT findings and an elevation of AFP. The tumors were categorized as "large" (>2 cm) in 44 cases and "small" (≤2 cm) in 28 cases, and as "multiple" (≥2 nodules) in 39 cases and "solitary" (single nodule) in 33 cases. Vascular invasion of the HCC was observed in 15 cases. According to the TNM staging of the Union Internationale Contre Le Cancer (UICC) classification system (6th version),³⁴ 30, 26, 9, 1, 2, and 4 patients were classified as having stages I, II, IIIA, IIIB, IIIC, and IV disease, respectively.

Selection of Potential HLA-A24–Binding Peptides Within hTERT. To identify potential HLA-A24–binding peptides, the amino acid sequences of hTERT were analyzed using a computer program designed to predict HLA-binding peptides based on the estimation of the half-time dissociation of the HLA–peptide complex. Ten peptides were selected according to the half-time dissociation scores (Table 2). Two of the 10 peptides have been reported to contain HLA-A*2402–restricted epitopes (peptides hTERT₄₆₁ and hTERT₃₂₄).³⁵ Next, MHC stabilization assays were performed to test the HLA-A*2402–binding capacity of these peptides using T2-A24 cells. Most peptides increased HLA-A24 expression, indicating that they bound and stabilized the HLA complex on the cell surface (Fig. 1). Peptide CMVpp65₃₂₈, which is identified as a strong binder of the HLA-A*2402 molecule,²⁵ also increased HLA-A24 expression. Percent MFI increase of the tested peptides except for peptides hTERT₁₀₀₉, hTERT₃₈₅, and hTERT₆₂₂ was greater than that of peptide AFP₁₃₇, which is HLA-A2 restricted.²⁶

Immunogenicity of hTERT Peptides in HLA-A*2402/K^b Transgenic Mice. To determine whether these HLA-A24–binding peptides include HLA-A*2402–restricted T cell epitopes, HLA-A*2402/K^b

transgenic mice were immunized with hTERT cDNA, and the spleen cell responses were evaluated by interferon gamma (IFN-γ) ELISPOT. Six of 10 hTERT-derived peptides were recognized by the spleen cells of at least one of the primed mice (Fig. 2). Peptides hTERT₁₀₀₉, hTERT₃₈₅, hTERT₆₂₂, and hTERT₈₆₉ were not recognized by any mice. These results show that peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇ may be immunogenic and contain the epitopes restricted by HLA-A*2402.

T Cell Responses to hTERT-Derived Peptides Assessed by IFN-γ ELISPOT Analysis in HCC Patients.

To determine whether these HLA-A24–binding peptides could be recognized by the T cells of patients with HCC, PBMC responses were evaluated by IFN-γ ELISPOT. Six of 10 hTERT-derived peptides were recognized by PBMCs of at least one patient, and 29 of 72 patients (40.3%) responded to at least one of the analyzed hTERT-derived peptides. An overview of all responses is shown in Fig. 3A. Single hTERT epitope-specific IFN-γ–producing cells were detected in 6 (8.3%), 6 (8.3%), 9 (12.5%), 5 (6.9%), 9 (12.5%), and 9 (12.5%) of 72 patients in response to the stimulation with peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇, respectively. Peptides hTERT₁₀₀₉, hTERT₃₈₅, hTERT₆₂₂, and hTERT₈₆₉ were not recognized by any patient.

The peptides recognized by PBMCs of the patients with HCC were comparable to those recognized by spleen cells of hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice. These peptides also displayed a relatively high affinity for the HLA-A*2402 molecule compared with the negative control peptide (Fig. 1). The strength of the hTERT-specific T cell responses assessed by the frequencies of IFN-γ–producing cells in the PBMC population is between 10 and 100 specific cells per 3 × 10⁵ PBMCs. Peptide CMVpp65₃₂₈, which includes an

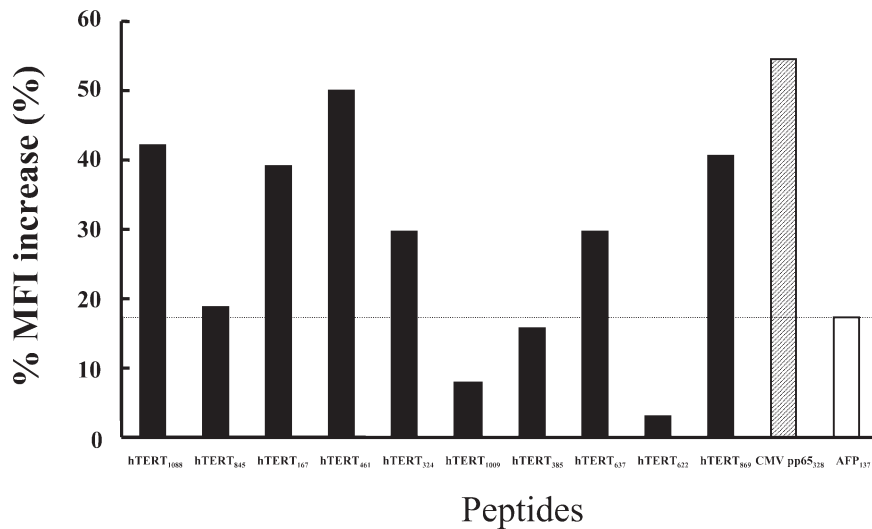


Fig. 1. MHC binding affinity. TAP-deficient T2-A24 cells were cultured for 16 hours at 26°C to enhance the expression of peptide-receptive cell surface molecules. They were incubated with individual peptides at 10 $\mu\text{g}/\text{mL}$ at 37°C for 2 hours, washed, and stained with anti-HLA-A24 monoclonal antibody, anti-mouse immunoglobulin-conjugated FITC, and 1 μg propidium iodide per milliliter. The data are expressed as the percent mean fluorescence intensity (MFI) increase for live, propidium iodide-negative cells. Peptide CMVpp65₃₂₈, a previously identified CMV pp65-derived peptide known to be a strong binder to HLA-A24, was used as a positive control. Peptide AFP₁₃₇, a previously identified AFP-derived peptide known to be HLA-A2 restricted, was used as a negative control. The experiment was performed three times, and a representative result is shown. MHC, major histocompatibility complex; HLA, human leukocyte antigen; FITC, fluorescein isothiocyanate; CMV, cytomegalovirus.

epitope derived from the CMV pp65 protein, and HCVNS3₁₀₃₁, which includes an epitope derived from the HCV NS3 protein, were also recognized by PBMCs of 31 of 72 (40%) and 12 of 51 (24%) patients with HCC, respectively. Conversely, no patients showed positive T cell responses against peptide HIVenv₅₈₄ derived from the HIV envelope protein, suggesting that these T cell responses were antigen-specific.

In contrast to the results for HCC patients, the ELISPOT assays for the healthy donors did not show more than 10 specific spots for all hTERT-derived peptides (Fig. 3B). The numbers of specific spots (mean \pm SD) in the healthy donors were 1.4 ± 1.7 , 0.6 ± 0.8 , 0.8 ± 1.1 , 0.7 ± 1.2 , 0.5 ± 0.7 , 0.6 ± 1.2 , 2.0 ± 2.6 , 1.7 ± 2.6 , 1.6 ± 3.4 , and 1.9 ± 2.9 for hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄,

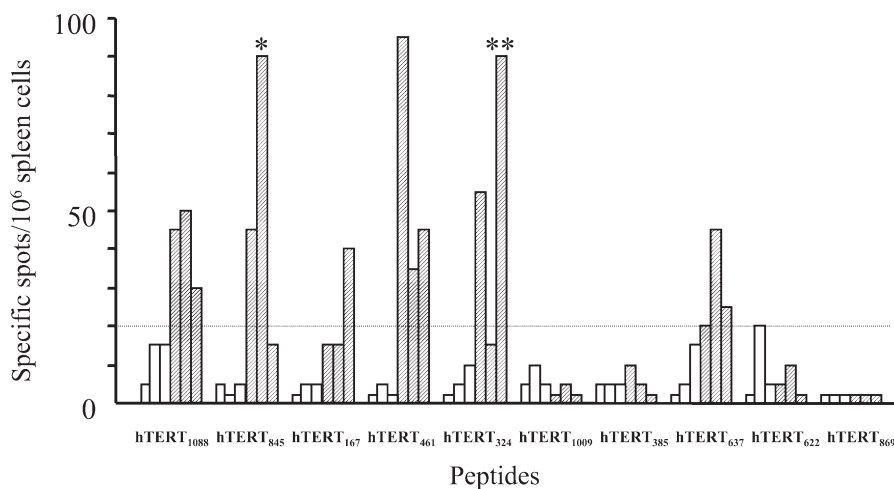


Fig. 2. Direct ex vivo analysis (IFN- γ ELISPOT assay) of spleen cell responses to hTERT-derived peptides in hTERT cDNA (hatched bars) or β -gal cDNA (open bars)-immunized HLA-A*2402/K^b transgenic mice. The immunization was performed in three mice for each cDNA. A positive T cell response was defined as more than 20 specific spots/ 1×10^6 spleen cells, which was the maximum response in β -gal cDNA-immunized mice. The peptide sequences are described in Table 2. * denotes 450 specific spots, ** denotes 130 specific spots. IFN- γ , interferon gamma; hTERT, human telomerase reverse transcriptase.

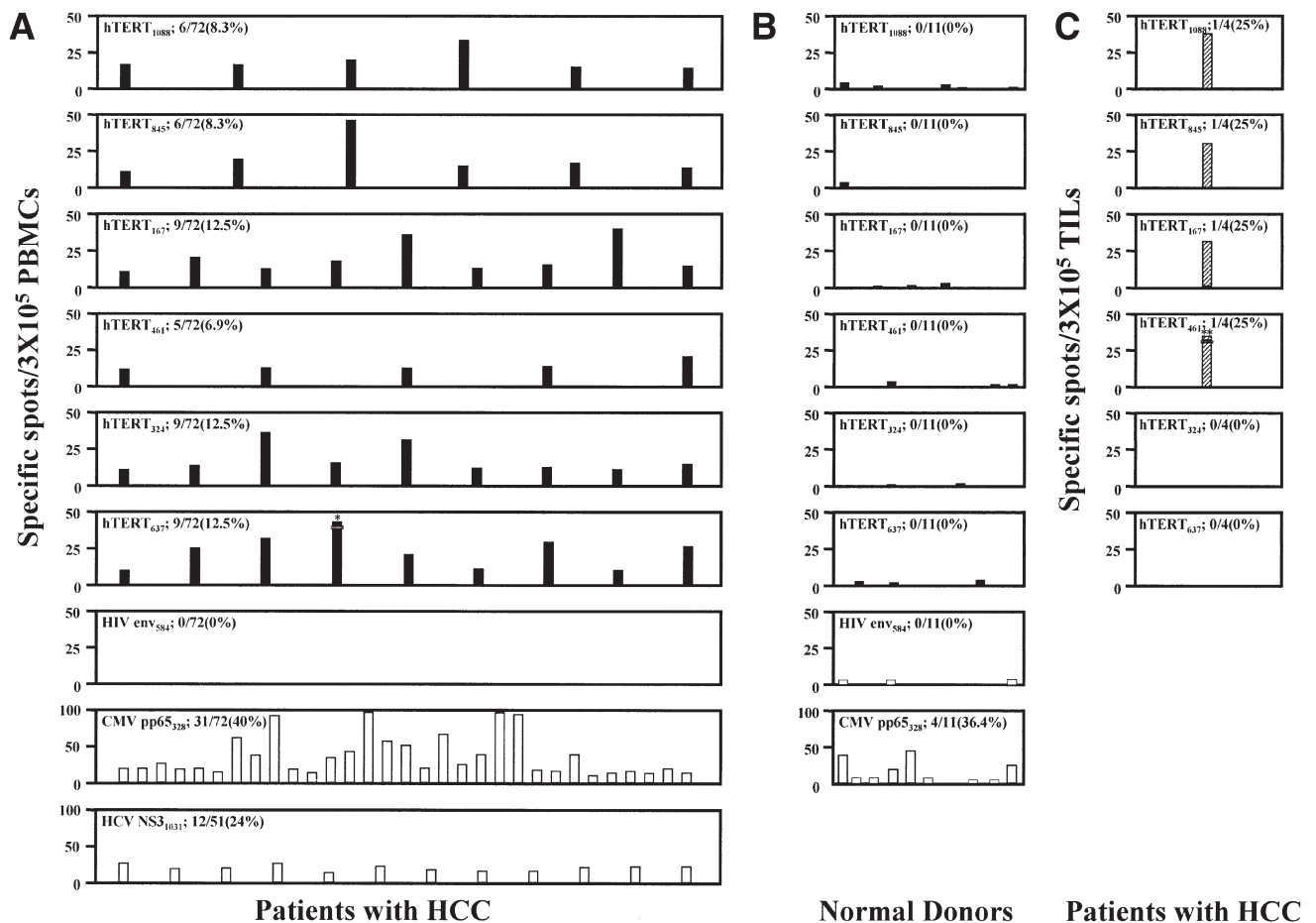


Fig. 3. Direct ex vivo analysis (IFN- γ ELISPOT assay) of peripheral blood T cell responses to hTERT-derived peptides (peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇; solid bars) or control peptides (Peptides HIVenv₅₈₄, CMVpp65₃₂₈ and HCVNS3₁₀₃₁; open bars) in HCC patients (A) and normal donors (B). Direct ex vivo analysis of tumor-infiltrating lymphocyte responses to hTERT-derived peptides (hatched bars) in HCC patients (C). Only significant IFN- γ responses are included in A and C. Responses were considered positive if more than 10 specific spots were detected and if the number of spots in the presence of antigen was at least twofold greater than that in the absence of antigen. The peptide sequences are described in Table 2. The data for peptides hTERT₁₀₀₉, hTERT₃₈₅, hTERT₆₂₂, and hTERT₈₆₉ are excluded because there was no positive T cell response. * denotes 100 specific spots. ** denotes 243 specific spots. IFN- γ , interferon gamma; hTERT, human telomerase reverse transcriptase; HCC, hepatocellular carcinoma.

hTERT₁₀₀₉, hTERT₃₈₅, hTERT₆₃₇, hTERT₆₂₂, and hTERT₈₆₉ peptides, respectively. The proportion of normal donors who showed positive T cell responses to CMV protein-derived peptides and the frequencies of the specific T cells were virtually the same as those of the HCC patients (Fig. 3B).

In ELISPOT assay using TILs, IFN- γ -producing T cells responding to peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, and hTERT₄₆₁ were detected as shown in Fig 3C, suggesting that hTERT-specific TILs were functional.

Cytotoxic Activity Against hTERT-Derived Peptides in HCC Patients. All hTERT-derived peptides were tested for their potential to induce HLA-A24-restricted CTLs from PBMCs of HCC patients with HLA-A24. Each peptide was tested on at least 10 patients. After three rounds of stimulation with the synthetic peptides, responder cells that had been stimulated with peptides hTERT₁₀₈₈,

hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇ lysed the peptide-pulsed C1R-A*2402 cells as shown in Fig. 4. Conversely, other peptides failed to induce CTLs specific for the corresponding peptides.

Cytotoxic Activity of hTERT Peptide-Specific CTLs Against Hepatoma Cell Lines. To examine whether hTERT peptide-specific CTLs induced from PBMCs of HCC patients lyse hepatoma cell lines that express hTERT, we first checked the telomerase activity in three hepatoma cells. TRAP assays showed that the three hepatoma cells expressed hTERT; however, the expression in HuH6 cells was lower than that in HepG2 or HuH7 cells (Fig. 5A). The results were confirmed in the TRAP ELISA, which is a quantitative measurement of telomerase activity. The expression levels of hTERT in HepG2 and HuH7 cells were more than twofold higher than the level in HuH6 cells (Fig. 5B).

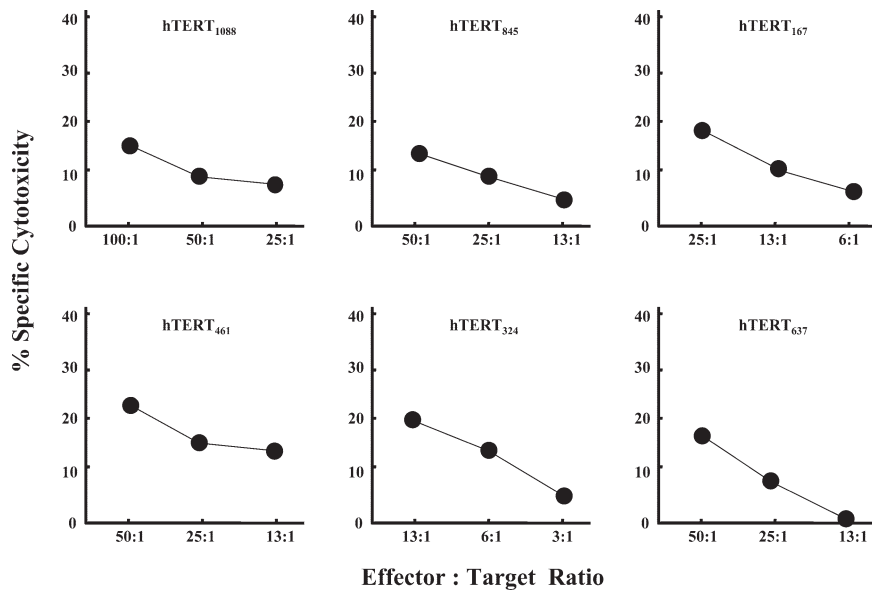


Fig. 4. Cytotoxicity of hTERT-specific T-cell lines derived with peptide in patients with HCC. The cytotoxicity of the T-cell lines was determined by a standard 6-hour cytotoxicity assay at various effector to target (E/T) ratios against C1R-A*2402 cells pulsed with one of the hTERT-derived peptides listed in Table 2. The data are indicated as the percent specific cytotoxicity, which is calculated as follows: (cytotoxicity in the presence of specific peptide) – (cytotoxicity in the absence of peptide). hTERT, human telomerase reverse transcriptase; HCC, hepatocellular carcinoma.

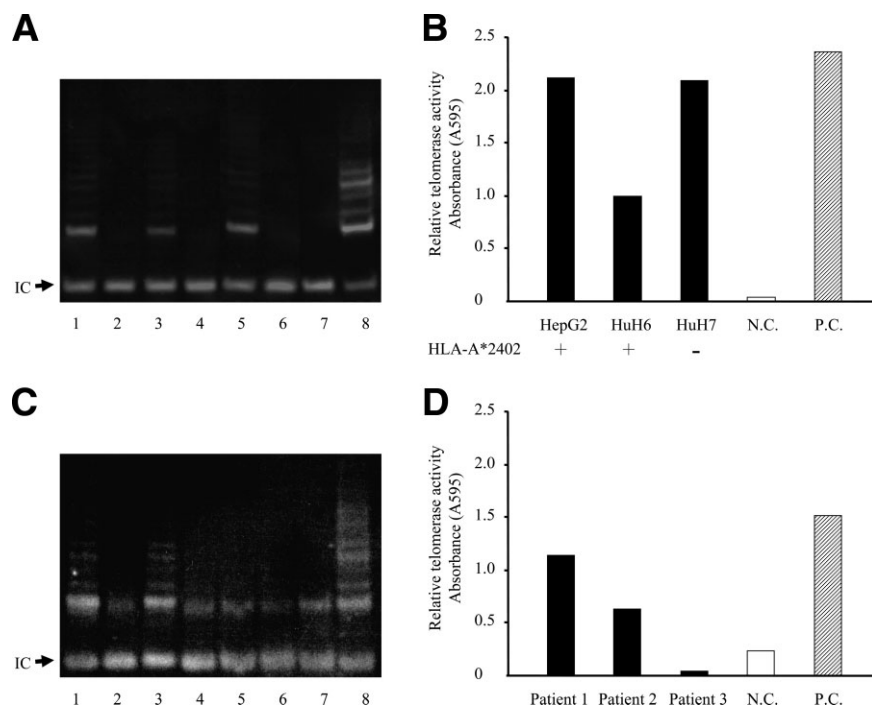


Fig. 5. Telomerase activity in hepatoma cell lines (A, B) and tumors resected by surgical treatment (C, D). A TRAP assay was carried out with 0.01 μ g and 0.1 μ g cell extract from hepatoma cell lines and tumors, respectively. The products of the PCR were fractionated by electrophoresis on a 10% polyacrylamide gel and then visualized by staining with SYBR-Green I. The TRAP internal control (IC) is shown for each extract. A: Lane 1; HepG2, Lane 2; HepG2 with heat, Lane 3; HuH 6, Lane 4; HuH 6 with heat, Lane 5; HuH 7, Lane 6; HuH 7 with heat, Lane 7; negative control, Lane 8; positive control. B: Lanes 1, 3, and 5, HCCs from three different patients; Lanes 2, 4, and 6, HCCs from three different patients with heat; Lane 7, negative control; Lane 8, positive control. Relative telomerase activity was measured with a TRAPEZE ELISA telomerase detection kit (TRAP ELISA) in hepatoma cell lines (C) and tumors resected by surgical treatment (D). Molecular typing of the HLA-A allele for hepatoma cell lines was performed with genomic DNA using standard site-specific oligonucleotide PCR. NC, negative control; PC, positive control; TRAP, telomerase repeat amplification protocol; PCR, polymerase chain reaction; HCC, hepatocellular carcinoma.

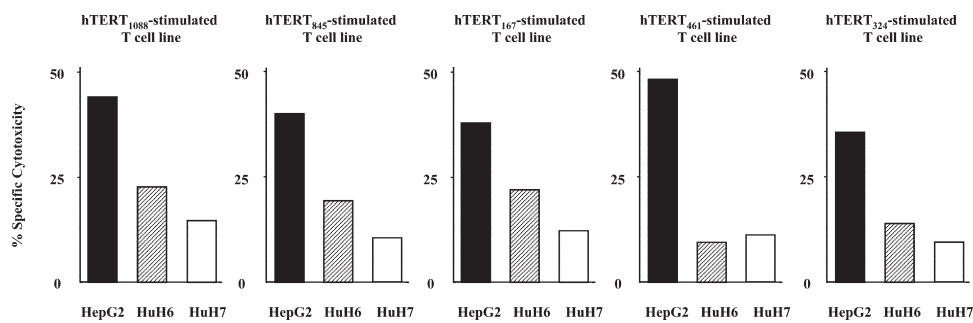


Fig. 6. Cytotoxicity of hTERT-specific T-cell lines derived with peptide against hepatoma cell lines. HepG2 (solid bar) highly expresses hTERT and has HLA-A*2402. HuH 6 (hatched bar) shows low expression of hTERT and has HLA-A*2402. HuH 7 (open bar) shows hTERT expression of the same level as HepG2 but does not have HLA-A*2402. The cytotoxicity was determined by a standard 6-hour cytotoxic assay (E/T ratio of 50:1). hTERT, human telomerase reverse transcriptase.

We next examined the cytotoxicity of hTERT peptide-specific CTLs against these hepatoma cell lines. As shown in Fig. 6, peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, and hTERT₃₂₄-specific CTLs showed cytotoxicity against HepG2 cells, which highly express hTERT and has the HLA-A*2402 molecule. In contrast, the CTLs did not show cytotoxicity against HuH7 cells, which express hTERT at the same level as HepG2 cells but do not have HLA-A*2402. In addition, the cytotoxicity of hTERT-specific CTLs induced with peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, and hTERT₃₂₄ against HuH6 cells, which express HLA-A*2402 and a low level of hTERT, was weak compared with the cytotoxicity against HepG2 cells. The difference was even more marked in the cytotoxicity of CTLs induced with peptide hTERT₄₆₁, and the CTLs were not cytotoxic to HuH6 cells.

Telomerase activity was also detected in the tumor of 3 of 10 patients with HCC (Fig. 5C and D). All of the three patients showed hTERT-specific T cell responses in ELISPOT assay.

Detection of hTERT₄₆₁ Tetramer⁺ and CD8⁺ T Lymphocytes in PBMCs and TILs. To analyze the character of hTERT specific T cells in patients with HCC more precisely, we examined the frequencies of hTERT₄₆₁ tetramer⁺ cells in PBMCs and TILs, and compared them with the results of ELISPOT assay. PBMCs and TILs were stained with CD4-FITC, CD14-FITC, CD19-FITC, CD8-PerCP, and tetramer-PE as described in Patients and Methods. At least 1 × 10⁵ cells in the CD8⁺CD4⁻CD14⁻CD19⁻ gate were then analyzed for tetramer staining as shown in Fig. 7A.

As indicated in Fig. 7B, the frequencies of CD8⁺CD4⁻CD14⁻CD19⁻hTERT₄₆₁ tetramer⁺ cells in peripheral blood were 0.03% to 0.71% of CD8⁺ T cells (patients 1-15). The frequencies in the patients with positive responses for ELISPOT assay were 0.06% to 0.71%. Interestingly, 7 of 10 patients without positive responses for ELISPOT assay showed 0.07% to 0.26% CD8⁺

CD4⁻CD14⁻CD19⁻hTERT₄₆₁tetramer⁺ cells. These results suggest that dysfunctional hTERT-specific T cells exist in patients with HCC. Conversely, the frequency of CD8⁺CD4⁻CD14⁻CD19⁻hTERT₄₆₁ tetramer⁺ cells in TILs was quite high (2.73%), and they were functional (patient 16).

hTERT-Specific T Cell Responses and Clinical Features of HCC Patients. To evaluate the status of hTERT-specific T cell responses in patients with HCC,

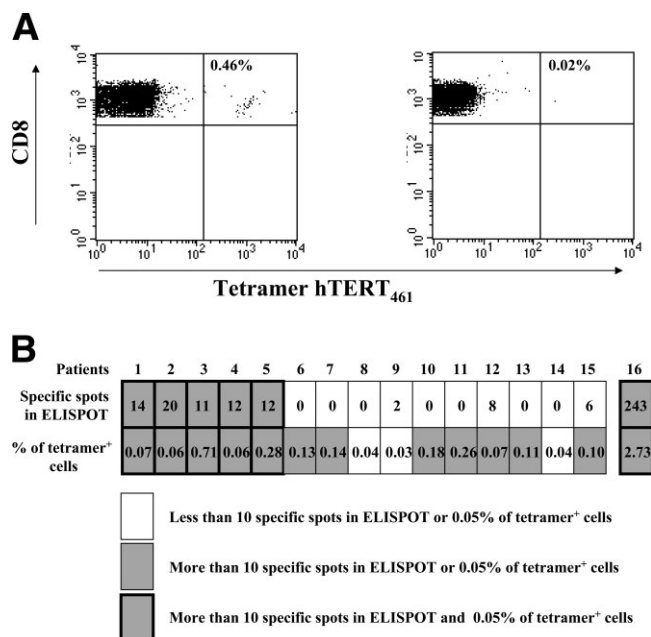


Fig. 7. Detection of hTERT-specific, HLA-A*2402-tetramer⁺, CD8⁺CD4⁻CD14⁻CD19⁻ T lymphocytes in the peripheral blood and tumor. PBMCs isolated from representative patients with HCC (A) were stained with tetrameric complexes and antibodies and analyzed on a FACSCalibur™ flow cytometer. Analysis of the association between the frequency of tetramer⁺ cells and IFN-γ-producing cells detected in ELISPOT assay (B). Tetramer staining and ELISPOT assay were performed in 16 patients using PBMCs (patients 1-15) and TILs (patient 16). hTERT, human telomerase reverse transcriptase; PBMC, peripheral blood mononuclear cell; HCC, hepatocellular carcinoma; IFN-γ, interferon gamma; TIL, tumor-infiltrating lymphocyte.

Table 3. Univariate Analysis of the Effect of Variables on the T Cell Response Against hTERT

	Patients With Positive T Cell Response	Patients Without Positive T Cell Response	P
No. of patients	29	43	
Age (years)*	67.7 ± 9.7	66.7 ± 8.1	NS
Sex (M/F)	21/8	27/16	NS
AFP level (≤20/> 20)	13/16	14/29	NS
Diff. degree of HCC (well/ moderate or poor/ND ^c)	9/6/14	6/16/21	NS
Tumor multiplicity (multiple/ solitary)	17/12	22/21	NS
Vascular invasion (+/-)	7/22	8/35	NS
TNM factor			
(T1/T2-4)	11/18	19/24	NS
(N0/N1)	28/1	43/0	NS
(M0/M1)	29/0	39/4	NS
TNM stage (I/II-IV)	11/18	19/24	NS
Histology of non-tumor liver (LC/Chronic hepatitis)	25/4	39/4	NS
Liver function (Child A/B/C)	13/14/2	30/11/2	NS
Etiology (HCV/HBV/Others)	22/3/4	37/6/0	NS

Abbreviations: NS; there was no statistical significance; ND, not determined.

*Data are expressed as mean ± SD.

we analyzed the relationship between the frequencies of peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇-specific T cells detected by IFN- γ ELISPOT assay and the clinical features of patients. Table 3 shows clinical features of HCC patients who showed positive and negative T cell responses to hTERT-derived epitopes.

The clinical features of both groups were not statistically different in terms of age, sex, serum AFP levels, differentiation of HCC, tumor multiplicity, vascular invasion, TNM factors and stages, histology of the non-tumor liver, liver function, and the type of viral infection (Table 3).

Next, we examined the kinetics of hTERT-specific T cells in 16 patients who had positive T cell responses and received curative treatments by surgical resection or radiofrequent ablation, and analyzed the association between the kinetics and clinical responses. The frequencies of hTERT-specific T cells detected in ELISPOT assay decreased in most of the patients 6 months after curative treatments (Fig. 8). Only 5 of 16 patients showed positive T cell responses after treatments. Four patients whose hTERT-specific T cells were maintained had no recurrence of HCC. In contrast, 11 patients whose number of hTERT-specific T cells decreased showed HCC recurrence within 1 year after curative treatments.

Discussion

In the current study, we first attempted to identify hTERT epitopes restricted by HLA-A24, which is present

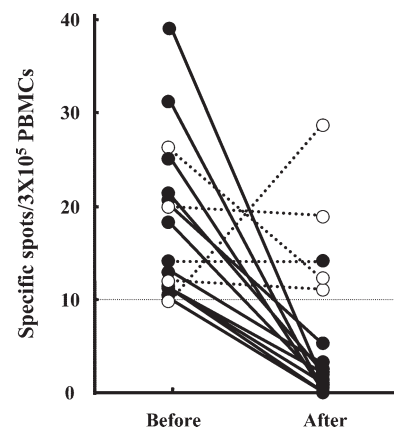


Fig. 8. Kinetics of hTERT-specific T cell responses before and after curative treatments. PBMCs were obtained before and 6 months after treatments and analyzed. Open circles show the patients without tumor recurrence within 1 year after treatment. Closed circles show the patients with tumor recurrence within 1 year after treatment. Solid and dotted lines show the patients without and with more than 10 specific spots for hTERT-derived peptides in ELISPOT assay after treatment, respectively. hTERT, human telomerase reverse transcriptase; PBMC, peripheral blood mononuclear cell.

in 60% of Japanese, 20% of whites, and 12% of Africans,^{36,37} using a combined computer-based and immunological approach. Analysis of amino acid sequences of hTERT by computer showed a number of potential HLA-A24-binding peptides, and 2 of the 10 hTERT-derived peptides (Peptides hTERT₄₆₁ and hTERT₃₂₄) have been identified to contain HLA-A24-restricted CTL epitopes. Including these two peptides, six hTERT-derived peptides (peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇) that showed high affinity for HLA-A*2402 induced production of IFN- γ in spleen cells and PBMCs, in hTERT cDNA-immunized HLA-A*2402/K^b transgenic mice and HCC patients, respectively. In addition, T cell lines stimulated with the peptide showed cytotoxicity against hepatoma cell lines that express HLA-A*2402 and hTERT. Taken together with the results of peptide binding, ELISPOT, and CTL assay, we concluded peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇ contained HLA-A24 restricted, hTERT-specific CTL epitopes.

Interestingly, the cytotoxicity of hTERT-specific CTLs induced with peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, and hTERT₃₂₄ in HuH6 cells, which showed low levels of hTERT, was weak compared with the cytotoxicity in HepG2 cells with high levels of hTERT. The difference was even more marked in the cytotoxicity of CTLs induced with peptide hTERT₄₆₁, and the CTLs were not cytotoxic to HuH6. In accordance with our results, it was reported that the susceptibility of tumor cells to hTERT-specific CTLs decreased after IFN- γ

treatment because of attenuation of hTERT expression.³⁸ In addition, all of the patients who had telomerase activity in the tumor showed hTERT-specific T cell responses in ELISPOT assay. These results suggest that the strength of hTERT-specific cytotoxicity against hepatoma cells depends on the expression levels of the protein.

In the analysis of PBMCs in patients with HCC using hTERT₄₆₁ tetramer, the frequencies of hTERT₄₆₁ tetramer⁺ cells in PBMCs were similar to those of other tumor specific antigen-derived epitopes.³⁹ Furthermore, the existence of dysfunctional hTERT-specific T cells was accordant with previous reports of other tumor antigens.³⁹ Conversely, the frequency of hTERT₄₆₁ tetramer⁺ cells in tumors was quite high, and they produced IFN- γ . IFN- γ -producing T cells responding to other peptides hTERT₁₀₈₈, hTERT₈₄₅, and hTERT₁₆₇ were also detected in tumors. These results suggest that hTERT is an attractive target for immunotherapy of HCC.

In the second part of the current study, to study the status of the host immunological response to hTERT in HCC patients, we examined the frequency of hTERT-specific T cells in the peripheral blood by ELISPOT assay with the six epitopes and analyzed the relationship between the frequency and the clinical features of the patients. ELISPOT assay showed that the frequency of reactive T cells to a single hTERT epitope was 10 to 100 per 3×10^5 PBMCs. In previous reports regarding the frequency of T cells specific for a single hTERT epitope in patients with colon or breast cancer, the number was found to be 1 to 22 per 2×10^5 PBMCs or 1 to 33 per 2×10^5 PBMCs, respectively.^{18,19} In addition, single hTERT epitope-specific IFN- γ -producing cells were detected in 6.9% to 12.5% of the patients for peptides hTERT₁₀₈₈, hTERT₈₄₅, hTERT₁₆₇, hTERT₄₆₁, hTERT₃₂₄, and hTERT₆₃₇. These rates are quite similar to those in previous reports.^{18,19} Comparing the current results with those reports, we believe that hTERT-specific CTL responses in HCC patients are as strong as those of other cancer patients and that the newly identified hTERT epitopes are immunogenic.

From the analysis of hTERT-specific immune responses in HCC patients, we obtained evidence that clinical features, including age, sex, serum AFP levels, differentiation of HCC, tumor multiplicity, vascular invasion, TNM factors and stages, histology of the non-tumor liver, liver function, and the type of viral infection, were not associated with the frequency of hTERT-specific CTLs in HCC patients (Table 3). These results suggest that hTERT-specific CTLs could be generated independently of hepatitis viral infection or serum AFP levels, which suppress the host immune response through inhibition of dendritic cells⁴⁰⁻⁴² or T cell proliferation.⁴³ In

addition, comparing with AFP- or other tumor antigen-specific immune responses,^{31,44} hTERT-specific immune responses exist and can be induced in the patients with HCC even at early stages. These results suggest the advantage of hTERT as a target for immunotherapies because the induction of tumor-specific immune responses at early stages of the tumor should be more effective for tumor growth suppression.

In the analysis of the association between kinetics of hTERT-specific T cells and clinical responses, recurrent rate of HCC was higher in the patients without maintenance of hTERT-specific T cells than in those with. This result suggests that maintenance of hTERT-specific T cells may be important to protect tumor recurrence after treatments, although there was no statistically significant difference between the two groups because of the small number of patients.

In conclusion, we identified and characterized HLA-A*2402-restricted T cell epitopes derived from hTERT. The identified epitope-specific T cells can be detected and induced by stimulating PBMCs with these peptides in HCC patients. hTERT-specific CTLs were observed even in the patients with early stages of HCC and killed hepatoma cell lines that expressed hTERT dependent on the expression level. The frequency of hTERT/tetramer⁺CD8⁺ T cells in the tumor tissue of patients with HCC was quite high, and they were functional. These results suggest that hTERT is an important target of T-cell-based immunotherapy for HCC and that the identified epitopes could be valuable both for therapy and for analyzing the host immune responses.

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